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Regulation of the human histamine H_1 receptor stably expressed in Chinese hamster ovary cells

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1 The human H_1 receptor gene expressed in Chinese hamster ovary cells ($CHOhumH_1$) encodes a classical histamine H_1 receptor with a pharmacology similar to that of the H_1 receptor found in guinea-pig cerebellum and the endogenously expressed human H_1 receptor in 1321N1 astrocytoma cells as determined by [^{3}H]-mepyramine binding studies.

2 In $CHOhumH_1$ cells, histamine induced a concentration-dependent rise in inositol phosphates (EC_{50} 2.23 ± 0.97 μM) and a rapid increase of $[Ca^{2+}]_i$, followed by a sustained increase of $[Ca^{2+}]_i$ upon addition of 100 μM histamine.

3 Short-term exposure of $CHOhumH_1$ cells to histamine (100 μM) resulted in a decrease of subsequent histamine-induced Ca^{2+} responses. The histamine-induced desensitization appeared to be heterologous as the ATP-induced Ca^{2+} response was also found to be affected.

4 The process of heterologous histamine-induced desensitization of the Ca^{2+} response in $CHOhumH_1$ cells can be ascribed to an alteration at the level of the intracellular Ca^{2+} pool, as the Ca^{2+} response of caffeine (10 mM), which releases Ca^{2+} from intracellular Ca^{2+} stores was also attenuated upon short-term histamine exposure.

5 In $CHOhumH_1$ cells the PKC activator, PMA, was found to inhibit the histamine (100 μM)-induced Ca^{2+} response concentration-dependently (IC_{50} 0.2 ± 0.03 μM) as well as the ATP (100 μM)-induced Ca^{2+} response. However, this inhibition was only partial and less effective than histamine-pretreatment. Moreover, in $CHOhumH_1$ cells PKC downregulation induced by long-term exposure to PMA (1 μM) did not affect the histamine-induced desensitization nor did pretreatment with the specific PKC inhibitor Ro-31-8220 (10 μM), indicating that in $CHOhumH_1$ cells PKC is probably not involved in the heterologous desensitization.

6 Long-term treatment of $CHOhumH_1$ cells with histamine or other H_1 agonists resulted in a time- and concentration-dependent decrease in the number of H_1 receptor binding sites (maximal reduction: $47 \pm 5\%$).

7 Long-term exposure of $CHOhumH_1$ cells to ATP or PMA did not affect H_1 receptor density.

8 Both histamine (100 μM)- and ATP (100 μM)-induced Ca^{2+} responses were affected upon long-term exposure of cells to histamine (100 μM), which might be explained by an alteration at a level distant from the receptor.

9 These results show that in $CHOhumH_1$ cells the human histamine H_1 receptor is susceptible to short-term and long-term receptor regulation in which PKC does not seem to play a role. The $CHOhumH_1$ cells therefore provide an excellent model system for studying the mechanism(s) of PKC-independent H_1 receptor regulation.

Keywords: Human histamine H_1 receptor; Chinese hamster ovary cells (CHO); [^{3}H]-mepyramine; desensitization; down-regulation; protein kinase C

Introduction

The gene encoding the histamine H_1 receptor was successfully cloned from the bovine adrenal medulla by means of an expression cloning strategy (Yamashita *et al.*, 1991). Soon thereafter, the guinea-pig (Horio *et al.*, 1993; Traiffort *et al.*, 1994), rat (Fujimoto *et al.*, 1993) and human homologues (De Backer *et al.*, 1993; Fukui *et al.*, 1994; Moguilevsky *et al.*, 1994) were cloned by homology screening. The cloning of these genes revealed that the histamine H_1 receptor belongs to the multigene family of G-protein-coupled receptors, which putatively all contain seven hydrophobic transmembrane domains, separated by hydrophilic intra- and extracellular loops (Collins, 1993).

For several members of the family of G-protein coupled receptors it has been shown that excessive stimulation of the receptor protein leads to a modulation of the receptor response and regulation of receptor expression (Collins, 1993; Lohse, 1993). Short-term activation of the histamine H_1 receptor is indeed frequently followed by a period of refractoriness, often referred to as desensitization. H_1 receptor desensitization has been observed and studied in several isolated tissues (Bristow *et al.*, 1993; Dillon-Carter & Chuang, 1989; Leurs *et al.*, 1990; Quach *et al.*, 1981) and cultured cell lines (Bristow & Zamani, 1993; Brown *et al.*, 1986; McDonough *et al.*, 1988; Dickenson & Hill, 1993; McCreath *et al.*, 1994; Smit *et al.*, 1992; Zamani *et al.*, 1994). Moreover, desensitization of H_1 receptor responses has also been observed *in vivo* (Poulakos & Gertner, 1986; Manning *et al.*, 1987; Antol *et al.*, 1988), indicating the importance of this process in physiology. So far no clear mechanistic details are known, although, depending on the cell type, protein kinase C (PKC)-dependent and independent

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mechanisms for short-term H_1 receptor desensitization have been observed (Dillon-Carter & Chuang, 1989; Cowlen *et al.*, 1990; Leurs *et al.*, 1990; Fukui *et al.*, 1991; Smit *et al.*, 1992; Dickenson & Hill, 1993; McCreath *et al.*, 1994). Similarly, both receptor specific (homologous)- and receptor non-specific (heterologous) desensitization have been observed in various cellular systems (Quach *et al.*, 1981; Brown *et al.*, 1986; McDonough *et al.*, 1988; Dillon-Carter & Chuang, 1989; Cowlen *et al.*, 1990; Leurs *et al.*, 1990; Smit *et al.*, 1992; Dickenson & Hill, 1993; McCreath *et al.*, 1994). Homologous desensitization is usually considered to be due to a specific modification of the receptor protein, whereas heterologous desensitization is caused by a modulation of a common signal transduction pathway.

Prolonged exposure of the G-protein coupled receptors to their respective agonist often results in a reduction in receptor number (Collins, 1993; Lohse, 1993). For the H_1 receptor, only one experimental study has been published, reporting a 20% downregulation of the H_1 receptor after an *in vitro* histamine treatment of guinea-pig brain tissue (Quach *et al.*, 1981). Yet, there are various reports that show that the H_1 receptor density is dynamically regulated *in vivo*. Yanai *et al.* (1992) showed by means of [^{11}C]-doxepin positron emission tomography (PET) studies that the H_1 receptor density in the brain decreases upon aging (Yanai *et al.*, 1992). Using the same technique Iinuma *et al.* (1993) revealed an upregulation of H_1 receptors in the electrical foci in the temporal cortex of epileptic patients (Iinuma *et al.*, 1993). Moreover, in the frontal cortex of patients with chronic schizophrenia a downregulation of H_1 receptors was reported (Nakai *et al.*, 1991). These examples indicate that the H_1 receptor expression is susceptible to regulatory changes under physiological and pathophysiological conditions, emphasizing the need to delineate further the mechanisms underlying the process of H_1 receptor regulation.

The recent cloning of the gene encoding the histamine H_1 receptor permits a more detailed investigation of the molecular mechanisms, related to the H_1 receptor function. Investigations of the downregulation of the H_1 receptor have been hampered so far by the lack of suitable model systems with a reasonable density of H_1 receptors. We therefore stably transfected the gene encoding the human H_1 receptor into Chinese hamster ovary (CHO) cells. Firstly, the expressed human H_1 receptor was subjected to a pharmacological characterization. Thereafter this cell line was used to investigate whether the expression of the human H_1 receptor can be modulated by prolonged exposure to histamine. Moreover, we also studied the effects of short-term exposure to histamine to evaluate the use of transfected CHO cells in mechanistic studies on homologous H_1 receptor desensitization. In all these studies the role of PKC in the regulatory processes was evaluated.

Methods

Transfection and cell culture

Chinese hamster ovary (CHO) cells, deficient in dihydrofolate reductase, were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2 in α-minimal essential medium (αMEM) with ribonucleosides and deoxyribonucleosides supplemented with L-glutamine (2 mM), penicillin (50 iu ml^{-1}), streptomycin (50 μg ml^{-1}) and 10% (v/v) foetal calf serum. CHO cells were stably transfected with the eukaryotic expression vector, pdKCR-dhfr containing the human H_1 receptor gene using the calcium phosphate precipitation method as described previously by Fujimoto *et al.* (1993). Selection was imposed by growing the cells in αMEM without ribonucleosides and deoxyribonucleosides supplemented with L-glutamine (2 mM), penicillin (50 iu ml^{-1}), streptomycin (50 μg ml^{-1}) and 10% (v/v) dialyzed foetal calf serum, which resulted in the appearance of several clonal cell lines. Thereafter, these cells were screened for expression of [^{3}H]-mepyramine binding.

CHO cells stably expressing the human H_1 receptor were cotransfected with the PKC isoenzymes α, β_I, β_{II}, δ, ε or γ cDNAs, inserted into a pTB vector (Ono *et al.*, 1987), and the pSV_2neo vector using the calcium phosphate method as described previously by Fujimoto *et al.* (1993). Neomycin-resistant clones were selected by growing the cells in medium containing G418 (500 μg ml^{-1}). The clones were screened by means of [^{3}H]-phorbol-12,13-dibutyrate binding.

The human 1321N1 human astrocytoma cells (Nakahata *et al.*, 1985) were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% foetal calf serum and supplemented with 2 mM L-glutamine, 50 iu ml^{-1} penicillin and 50 μg ml^{-1} streptomycin.

Membrane preparation

CHOhumH_1 cells and 1321N1 cells were harvested by means of a cell scraper and recovered by a 10 min centrifugation at 500 *g*. Cells were homogenized in ice-cold 50 mM Na_2/K-phosphate buffer (pH = 7.4) with a Polytron homogenizer (5 s, maximal speed) and used for radioligand binding studies. Protein concentrations were determined according to Bradford (1976) with bovine serum albumin used as a standard.

Histamine H_1 receptor binding

Membranes (60–80 μg of protein) were incubated for 30 min at 25°C in 50 mM Na_2/K phosphate buffer in a total volume of 400 μl with the indicated concentrations of [^{3}H]-mepyramine. In saturation studies, increasing concentrations of [^{3}H]-mepyramine were incubated with the membranes in the absence or presence of 1 μM mianserin. In displacement studies, membranes were incubated with 2 nM [^{3}H]-mepyramine and increasing concentrations of competing ligands. The incubations were stopped by rapid dilution with 3 ml ice-cold 50 mM Na_2/K phosphate buffer (pH = 7.4). The bound radioactivity was subsequently separated by filtration with a Brandel cell harvester (Semat, UK) through Whatman GF/B glass fibre filters that had been treated with 0.3% polyethyleneimine. Filters were washed twice with 3 ml buffer and radioactivity retained on the filters was measured by liquid scintillation counting. The binding data were evaluated by use of LIGAND, a non-linear, weighted, least squares curve-fitting procedure (Munson & Rodbard, 1980).

Changes in H_1 receptor density were denoted as a percentage downregulation compared to non-treated control cells. During the 24 h incubation of cells with various histamine ligands or other compounds, cells were grown in serum-free medium.

[^{3}H]-inositol phosphate measurements

CHOhumH_1 cells were seeded in 12-well plates and cultured overnight in culture medium. Thereafter, cells were labelled overnight in inositol-free medium supplemented with 1 μCi ml^{-1} [^{3}H]-*myo*-inositol. Cells were washed twice with Krebs buffer composition, mM: NaCl 125, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11, HEPES 15, $NaHCO_3$ 15, pH = 7.4 at 37°C), supplemented with 10 mM LiCl, and preincubated for 10 min at 37°C with or without one of the histamine receptor antagonists at the indicated concentrations. Incubations were started by the addition of histamine. After 10 min incubation at 37°C the medium was aspirated and the reaction was stopped by addition of 5% TCA. The cells were chilled on ice for 10 min. Thereafter, 2 ml of water-saturated diethylether was added to the TCA extract and was mixed for 5 min. The ether phase was aspirated and the procedure was repeated once more. After aspiration the remaining diethylether was removed by incubation at 37°C; 150 μl 0.2 M Tris-HCl was then added to neutralize the samples and the

[^{3}H]-inositol phosphates were isolated by anion exchange chromatography (Godfrey, 1992).

Measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

CHOhumH_1 cells were trypsinized, washed with αMEM supplemented with 0.2% bovine serum albumin (BSA) and resuspended in αMEM/0.2% BSA containing 2 μM fura-2-AM and 0.025% Pluronic-F-127 for 1 h at 25°C. Thereafter cells were gently washed with 5 ml of αMEM/0.2% BSA, resuspended in 1 ml of αMEM/0.2% BSA and stored on ice. A 100 μl sample was added to 1.5 ml prewarmed Tyrode solution (composition mM: $CaCl_2$ 2.5, KCl 2.7, $MgCl_2$ 0.5, NaCl 137, NaH_2PO_4 0.36, glucose 5.6 and HEPES 10, pH = 7.4) in a temperature-controlled (30°C) quartz cuvette. Ca^{2+}-dependent fura-2 fluorescence was monitored at an emission wavelength of 510 nm with an excitation wavelength of 340 and 380 nm in a Shimadzu RF5001PC. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the formula derived by Grynkiewicz *et al.* (1985). Maximal fluorescence was measured (F_{max}) by permeabilization of cells with 0.2% Triton-X-100 and minimal fluorescence (F_{min}) was achieved by complexing calcium with 10 mM EGTA. For the calculation of the intracellular Ca^{2+} concentration, the fluorescence values were corrected for autofluorescence.

All desensitization experiments were carried out with CHOhumH_1 cells grown on a coverslip. In these experiments cells were loaded with 2 μM fura-2-AM in a HBS buffer (composition, mM: NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, 0.2% BSA, HEPES 10, pH = 7.4) for 1 h at 25°C. Thereafter, the coverslip was inserted into the quartz cuvette and cells were washed by completely exchanging prewarmed HBS buffer (30°C). Fluorescence was measured as described above.

[^{3}H]-phorbol-12,13-dibutyrate binding

CHOhumH_1 cells were seeded in 12-well plates and cultured overnight in culture medium. Cells were washed with PBS and preincubated for 10 min in αMEM supplemented with 0.1% bovine serum albumin and 20 mM HEPES (pH = 7.0) (binding buffer) at 37°C. Thereafter, cells were incubated in binding buffer with 5 nM [^{3}H]-phorbol-12,13-dibutyrate in the presence or absence of 10 μM PMA for 1 h at 37°C. The incubations were stopped by washing with ice-cold PBS buffer supplemented with 0.1% bovine serum albumin. Next, cells were incubated with trypsin-EDTA for several hours in order to disrupt the cells. The bound radioactivity was subsequently separated by filtration through GF/B glass fibre filters. Filters were washed with cold PBS and the radioactivity retained on the filters was counted. The difference in radioactivity measured in cells incubated in the absence and presence of 10 μM PMA was considered as specific phorbol ester binding.

Chemicals

Histamine dihydrochloride, phorbol-12-myristate-13-acetate, 4α-phorbol, bovine serum albumin (BSA), caffeine and ATP (disodium salt) were obtained from Sigma Chemical Company (U.S.A.). Ro-31-8220 was purchased from Calbiochem (U.S.A.). Dowex AG1x8 (200-400 mesh) formate form was obtained from Bio-rad (Bio-rad laboratories GmbH, Germany). [^{3}H]-mepyramine ([pyridinyl-5-^{3}H]pyrilamine, 21 Ci mmol^{-1}), [^{3}H]-phorbol-12,13-dibutyrate (15.1 Ci mmol^{-1}) and [^{3}H]-*myo*-inositol (18.8 Ci mmol^{-1}) were purchased from Amersham. Fura-2-acetoxymethylester (fura-2-AM) and Pluronic-F-127 were obtained from Molecular Probes (Eugene, OR, U.S.A.). 2-Pyridylethylamine dihydrochloride was taken from laboratory stock. Gifts of mianserin (Organon, The Netherlands), 2-methylhistamine dihydrochloride, 2-thiazolylethylamine dihydrochloride (SmithKline Beecham, United Kingdom), the enantiomers of cicletanine (Institute of Henri Beaufour, France) and the enantiomers of chlorpheniramine (maleate salts) (Dr A. Belt, Nijmegen, The Netherlands), are gratefully acknowledged.

Statistical analysis

All data shown are expressed as mean ± standard errors (mean ± s.e.) of at least three independent experiments. Statistical analysis was carried out by Student's *t* test. *P*-values < 0.05 were considered statistically significant; *n* in the text refers to the number of separate experiments.

Results

Pharmacological characterization of the human histamine H_1 receptor expressed in Chinese hamster ovary cells (CHOhumH_1)

Transfection of the cDNA encoding the human H_1 receptor (Fukui *et al.*, 1994) into CHO cells resulted in the isolation of several clones, expressing [^{3}H]-mepyramine binding sites. Untransfected cells did not show any specific [^{3}H]-mepyramine binding. One clonal cell line (CHOhumH_1) was selected for further analysis. Saturation experiments performed with CHOhumH_1 cell membranes revealed the presence of a single population of saturable [^{3}H]-mepyramine binding sites with a dissociation constant (K_d) of 1.10 ± 0.09 nM and a maximal

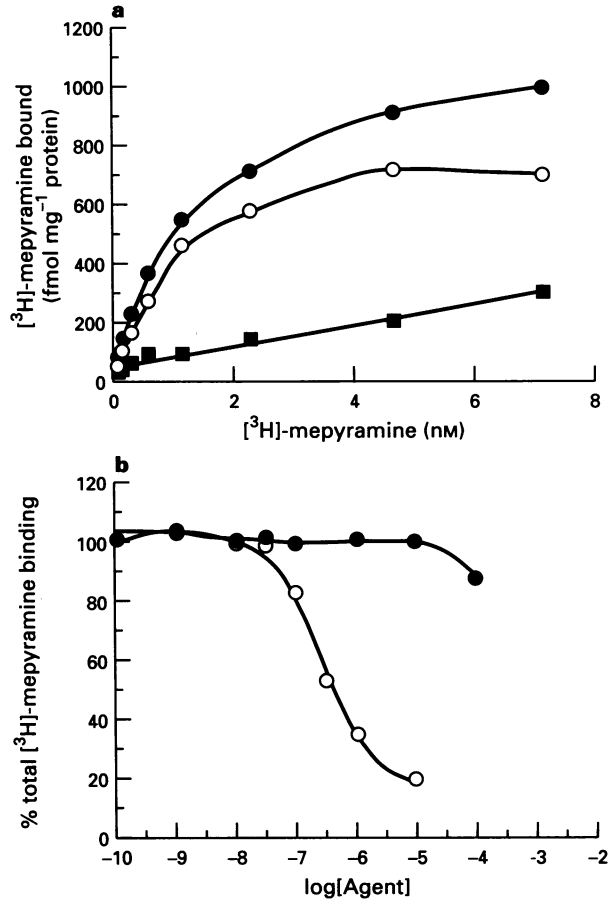

Figure 1 Binding of [^{3}H]-mepyramine to CHOhumH_1 cell membranes. (a) Saturation binding of [^{3}H]-mepyramine to CHOhumH_1 cell membranes. Specific radioligand binding (○) was determined by subtracting the binding determined in the presence of 1 μM mianserin (■) from the total binding (●). (b) Displacement of binding of 2 nM [^{3}H]-mepyramine by increasing concentrations of (−)-cicletanine (○) and (+)-cicletanine (●). Mean ± s.e. values of triplicate determinations of a typical experiment out of three are shown.

density (B_{max}) of 861±41 fmol mg^{-1} protein (n=3, mean±s.e.) (Figure 1a). The binding of 2 nM [^{3}H]-mepyramine to CHOhumH$_1$ cell membranes was monophasically and stereoselectively inhibited by various H$_1$ antagonists (Figure 1b, Table 1). For comparison, binding studies were performed on membranes of human 1321N1 astrocytoma cells, which endogenously express a low level of H$_1$ receptors (Nakahata *et al.*, 1985). Saturation experiments revealed a K_d value of [^{3}H]-mepyramine of 1.14±0.22 nM and a B_{max} of 131±46 fmol mg^{-1} protein (n=3, mean±s.e.). The pK_i values for the different H$_1$ antagonists on CHOhumH$_1$ membranes were found to be consistent with the observed pK_i values on 1321N1 membranes and reported pK_i values determined on guinea-pig cerebellum (Ter Laak *et al.*, 1993) (Table 1). Histamine displaced the specific [^{3}H]-mepyramine binding to CHOhumH$_1$ membranes with a pK_i value of 4.67±0.02 (n=5, mean±s.e.), a value that closely corresponds to the pK_i-value previously determined with 1321N1 astrocytoma membranes (Nakahata *et al.*, 1985).

Experiments with [^{3}H]-*myo*-inositol labelled CHOhumH$_1$ cells showed a concentration-dependent increase in inositol phosphate production when cells were incubated for 10 min with increasing concentrations of histamine (Figure 2a). The EC_{50} value of histamine for this response was 2.23±0.97 μM (n=3, mean±s.e.). A 10 fold stimulation over basal levels of inositol phosphate production was observed when cells were stimulated with 100 μM histamine. The H$_1$ antagonist, mepyramine (1 μM), inhibited the histamine (100 μM)-induced production of inositol phosphates (62%), whereas the H$_2$ antagonist, ranitidine (10 μM) and the H$_3$ antagonist, thioperamide (1 μM) did not affect the histamine-induced inositol phosphate response (Figure 2a inset).

Addition of 100 μM histamine to fura-2AM-loaded CHOhumH$_1$ cells resulted in a rapid increase of the intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) (basal [Ca^{2+}]$_i$: 91±4 nM; histamine-induced increase [Ca^{2+}]$_i$: 1040±53 nM, n=8, mean±s.e.) (Figure 2b). The rapid transient increase was followed by a sustained increase in [Ca^{2+}]$_i$ (477±27 nM) lasting until the agonist was removed. In contrast, with the partial inhibition of mepyramine of the histamine-induced inositol phosphate production, preincubation of these cells with 1 μM mepyramine for a period of 10 min prior to stimulation with histamine (100 μM) totally blocked the histamine-induced increase of [Ca^{2+}]$_i$ (Figure 2b).

Short-term desensitization of the human histamine H_1 receptor expressed in CHO cells

Desensitization of the histamine-induced Ca^{2+} response in CHOhumH$_1$ cells was observed when the same cells were repeatedly exposed to histamine (Figure 3). After the first challenge with 100 μM histamine for a period of 5 min, cells were washed for 5 min and re-exposed to 100 μM histamine for another 5 min. Thereafter, this procedure was repeated once

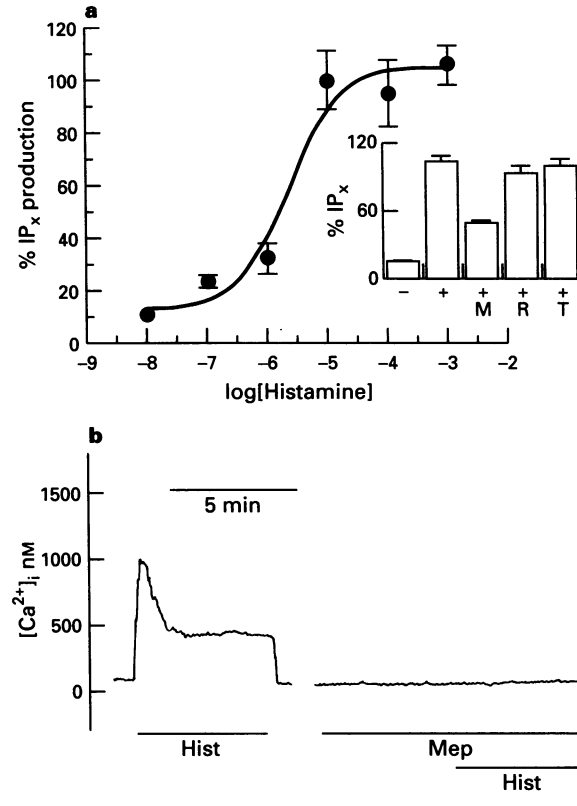

Figure 2 Signal transduction pathways of the human H$_1$ receptor expressed in CHO cells. (a) Histamine-induced production of [^{3}H]-inositol phosphates. Cells were prelabelled overnight with [^{3}H]-*myo*-inositol, were washed twice and preincubated for 10 min in αMEM containing 10 mM LiCl. Thereafter, cells were incubated for 10 min with increasing concentrations of histamine in the same medium. (basal inositol phosphate levels: 2127±371 d.p.m./well, histamine induced increases of inositol phosphate production: 23449±2872 d.p.m./well, n=3, mean±s.e.). Mean±the s.e. values of triplicate determinations of a typical experiment out of three are shown. (Inset) Effect of the histamine H$_1$ antagonist mepyramine (M) (1 μM), H$_2$ antagonist ranitidine (R) (10 μM) and H$_3$ antagonist thioperamide (T) (1 μM) on the histamine-induced increase in inositol phosphate production over basal levels (−) (n=2). Cells were preincubated for 10 min with the antagonists. Thereafter, cells were stimulated with 100 μM histamine (+). (b) Histamine-induced increase in [Ca^{2+}]$_i$ in fura-2/AM-loaded CHOhumH$_1$ cells grown on a coverslip. Cells were incubated at 30°C in HBS buffer and exposed to 100 μM histamine for 5 min in the absence or the presence of 1 μM mepyramine (10 min preincubation) and washed with HBS buffer. Data shown are from two different representative experiments (basal [Ca^{2+}]$_i$: 91±4 nM: histamine-induced increase [Ca^{2+}]$_i$: 1040±53 nM, n=8, mean±s.e.).

Table 1 Characterization of [^{3}H]-mepyramine binding to CHOhumH$_1$ cell membranes

Antagonists	*pK_i*		
	Guinea-pig cerebellum	*1321N1 cells*	*CHOhumH$_1$ cells*
(+)-Cicletanine	<5	<5	<5
(−)-Cicletanine	7.54	7.74±0.04	7.27±0.06
(+)-Chlorpheniramine	8.32	8.35±0.20	8.45±0.04
(−)-Chlorpheniramine	6.68	6.78±0.12	6.53±0.06

Membranes of CHOhumH$_1$ cells and 1321N1 cells were incubated with 2 nM [^{3}H]-mepyramine in the presence of the indicated drugs at increasing concentrations. K_i values were obtained from the respective IC_{50} values. Data shown are the mean±s.e. from three independent experiments. Reported values for [^{3}H]-mepyramine binding to guinea-pig cerebellum (Ter Laak *et al.*, 1993) are shown for comparison.

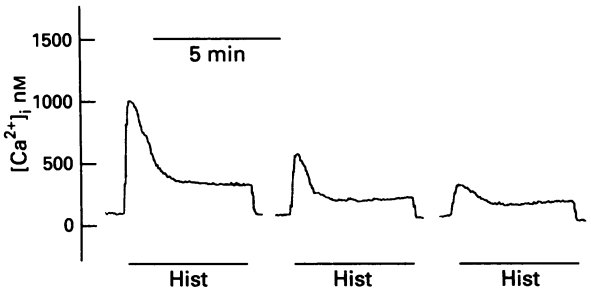

Figure 3 Desensitization of the histamine-induced Ca^{2+} transients in CHOhumH$_1$ cells. Cells were repeatedly exposed to 100 μM histamine (Hist) for a period of 5 min. After each histamine challenge cells were washed for 5 min by completely exchanging the buffer. A typical experiment out of 6 is shown.

more. In total therefore, three histamine-induced Ca^{2+} responses were recorded. As depicted in Figure 3 the consecutive responses are markedly affected by the previous challenges to histamine (basal $[Ca^{2+}]_i$: 91±4 nM; first histamine-induced increase $[Ca^{2+}]_i$: 1040±53 nM, $n=8$, second histamine-induced increase $[Ca^{2+}]_i$: 458±60 nM (reduction 56±6%, $n=6$), third histamine-induced increase $[Ca^{2+}]_i$: 169±12 nM (reduction of 84±1%, $n=6$, mean±s.e.)).

In CHOhumH$_1$ cells, 100 μM ATP also induced a rapid rise of the $[Ca^{2+}]_i$, followed by a sustained increase in $[Ca^{2+}]_i$ (basal $[Ca^{2+}]_i$ 79±11 nM, ATP-induced increase $[Ca^{2+}]_i$: 1489±131 nM, $n=3$, mean±s.e.). Pretreatment of these CHOhumH$_1$ cells with 100 μM histamine for 5 min also affected the Ca^{2+} response induced by 100 μM ATP. A decrease of 63±5% ($n=4$, mean±s.e.) was observed when CHOhumH$_1$ cells were pretreated for 5 min with histamine. Preincubation of cells with 100 μM ATP for 5 min also resulted in a 66±3% (mean±s.e., $n=3$) attenuation of the histamine-induced Ca^{2+} response. Caffeine (10 mM) induced a rise in $[Ca^{2+}]_i$ in CHOhumH$_1$ cells (332±23 nM, $n=4$, mean±s.e.). After a 5 min pretreatment of cells with 100 μM histamine which was followed by a 5 min washing period, the caffeine response was significantly reduced to 45±5% of the control response (mean±s.e., $n=3$).

Role of protein kinase C in the process of short-term desensitization

Pretreatment of CHOhumH$_1$ cells for 10 min with increasing concentrations of the PKC activator, PMA, resulted in a maximum decrease of 55±5% (mean±s.e., $n=3$) (Figure 4a) of the histamine-induced Ca^{2+} response, whereas pretreatment with the inactive phorbol ester, 4α-phorbol did not affect the histamine-induced Ca^{2+} response (4α-phorbol-treated cells: 98±9% vs control response, mean±s.e., $n=4$, Figure 4a). The IC_{50} value of the effect of PMA was 0.2±0.03 μM ($n=3$, mean±s.e.). The effect of PMA was maximal at a concentration of 1 μM and after a 10 min incubation as was found in previous studies performed by Leurs *et al.* (1994). The ATP-induced Ca^{2+} response was also found to be inhibited by 40±2% upon pretreatment with 1 μM PMA (Figure 4a).

The specific PKC inhibitor Ro-31-8220 (10 μM) blocked the PMA (1 μM)-induced reduction of the histamine-induced Ca^{2+} response (Figure 4b) without affecting the histamine (100 μM)-induced Ca^{2+} response. Exposure of cells to the specific PKC inhibitor, Ro-31-8220 (10 μM) for 20 min prior to and during histamine pretreatment did not inhibit the histamine-induced desensitization of the histamine-induced Ca^{2+} response (control cells: 56±4% desensitization, $n=6$, Ro-31-8220-treated cells: 63±6% desensitization, $n=4$, mean±s.e.) (Figure 4b).

In addition, the histamine-induced Ca^{2+} response was further reduced when cells were preincubated with histamine (100 μM) and PMA (1 μM) together (PMA-treated cells: 55±5% reduction; PMA/histamine-treated cells: 85±7% reduction), again indicating that the histamine-induced desensitization and PMA-induced desensitization are separate processes.

Finally, CHOHumH$_1$ cells were incubated for 18 h with 1 μM PMA to downregulate PKC. Control experiments in which PKC downregulated cells were subsequently pretreated with 1 μM PMA for 10 min still showed a reduction of 22±2% (mean±s.e., $n=6$) of the histamine-induced Ca^{2+} response. However, PKC downregulation by 18 h incubation of cells with 1 μM PMA did not affect the histamine-induced desensitization (control cells: 56±4% desensitization, PKC downregulated cells: 66±9% desensitization, $n=6$, mean±s.e.) (Figure 4c). Long-term (18 h) incubation of the CHOhumH$_1$ cells with 10 μM PMA also did not inhibit the histamine-induced desensitization (data not shown).

Long-term desensitization of the human histamine H_1 receptor expressed in CHO cells

Incubation of CHOhumH$_1$ cells with 100 μM histamine for periods ranging from 2 to 32 h resulted in a time-dependent decrease of [^{3}H]-mepyramine binding (Figure 5a). A maximal reduction ($n=3$, mean±s.e.) was observed after 24 h incubation of cells with 100 μM histamine. Half-maximal decrease of [^{3}H]-mepyramine binding was recorded after an incubation period of about 14 h. Saturation studies showed that the K_d value of [^{3}H]-mepyramine was not affected by 24 h incubation of cells with 100 μM histamine (K_d of [^{3}H]-mepyramine in control cells: 1.10±0.09 nM; histamine-treated cells: 0.98±0.10 nM). Long-term exposure (24 h) of CHOhumH$_1$ cells with 100 μM histamine resulted in a reduction (47±5%, $n=3$, mean±s.e.) of the total number of [^{3}H]-mepyramine binding sites (B_{max}) (B_{max} in control cells: 861±41 fmol mg^{-1} of protein; histamine-treated cells: 460±46 fmol mg^{-1} of protein). The decrease in [^{3}H]-mepyramine binding was shown to be concentration-dependent, as 24 h incubation of CHOhumH$_1$ cells with increasing concentrations of histamine resulted in a concentration-dependent reduction of [^{3}H]-mepyramine binding, with an EC_{50} of 0.13±0.02 μM ($n=3$, mean±s.e.) (Figure 5b). Exposure of CHOhumH$_1$ cells for 24 h to histamine H$_1$ agonists, such as 2-pyridylethylhistamine (2-PEA), 2-methylhistamine (2-MeHA) and 2-thiazolylethylamine (2-TEA) also induced a significant reduction of [^{3}H]-mepyramine binding (inset Figure 5b).

Role of PKC in the process of histamine-induced H_1 receptor downregulation

Exposure of CHOhumH$_1$ cells for 24 h to 0.1 μM or 1 μM PMA did not affect [^{3}H]-mepyramine binding (Table 2). Incubations of CHOhumH$_1$ cells with 1 μM PMA for shorter periods of time (4 h) also did not affect [^{3}H]-mepyramine binding (data not shown). Similar observations were made for incubation of cells with 1 μM PdBu. Concurrent exposure of CHOhumH$_1$ cells to 100 μM histamine and 1 μM PMA for 24 h caused a significant reduction of [^{3}H]-mepyramine binding, which was significantly less pronounced than treatment with histamine alone (Table 2). Co-incubation of cells with PMA (1 μM) and histamine (100 μM) for 4 h did not result in a reduction of [^{3}H]-mepyramine binding (data not shown). Exposure of CHOhumH$_1$ cells to 100 μM ATP for 24 h also did not induce a change in the number of [^{3}H]-mepyramine binding sites (Table 2).

Effect of long-term desensitization on histamine- and ATP-induced Ca^{2+} signalling

A marked attenuation of the 100 μM histamine-induced Ca^{2+} response was observed when the CHOhumH$_1$ cells were previously exposed to 100 μM histamine for 24 h (reduction of 57±4%, $n=4$, mean±s.e.). This treatment also impaired the ATP-induced rise (100 μM) in Ca^{2+} (reduction 32±6%, $n=4$, mean±s.e.).

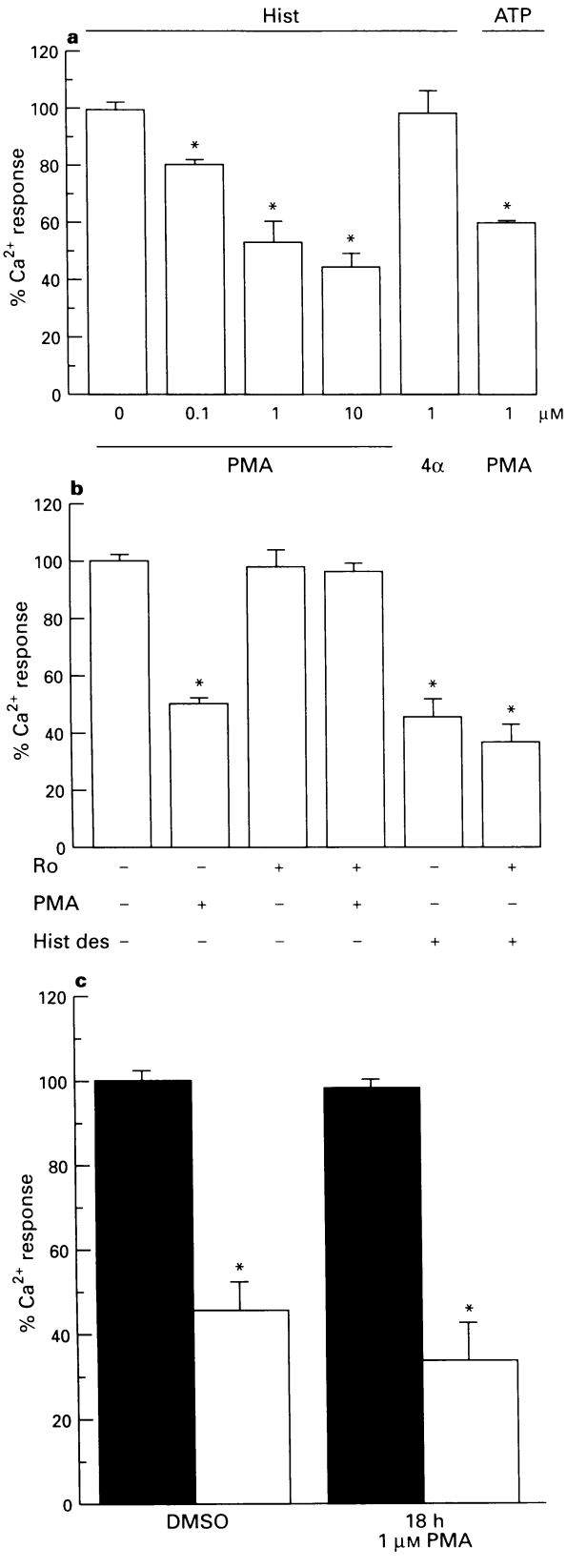

Figure 4 Effect of the PKC activator, PMA and the PKC inhibitor, Ro-31-8220 on the histamine-induced Ca^{2+} response and histamine-induced desensitization in CHOhumH$_1$ cells. (a) Effect of increasing concentrations of PMA and 1 μM 4α-phorbol (4α) on the histamine-induced Ca^{2+} response (Hist). CHOhumH$_1$ cells were pretreated for 10 min with the indicated concentrations of PMA or with 1 μM 4α-phorbol for 10 min and exposed to 100 μM histamine for 5 min in the presence of the respective PMA/4α-phorbol concentration. The same experimental procedure was used for the determination of the effect of 1 μM PMA pretreatment on the ATP (100 μM)-induced Ca^{2+} response. The data shown represent the mean$\pm$s.e. at least 3 independent experiments. (b) Effect of Ro-31-8220 on the histamine-induced Ca^{2+} response and histamine-induced desensitization. CHOhumH$_1$ cells were pretreated with or without 10 μM Ro-31-8220 (Ro) for 20 min and with or without 1 μM PMA for 10 min and exposed to 100 μM histamine in the presence of the pretreating agent(s). In the desensitization experiments CHOhumH$_1$ cells were pretreated with 10 μM Ro-31-8220 for 20 min and exposed twice to 100 μM histamine. Only the second response to histamine (Hist des) is depicted in this figure. Cells were washed for 5 min after the first exposure to histamine. The data shown represent the mean$\pm$s.e. of 4 independent experiments. (c) Effect of 18 h treatment of CHOhumH$_1$ cells with 1 μM PMA on the histamine-induced desensitization. Control (DMSO) and PMA-treated (18 h) cells were repeatedly exposed to 100 μM histamine for a period of 5 min (first histamine response: solid columns; second histamine response: open columns). After the first histamine challenge cells were washed for 5 min by completely exchanging the buffer. The data shown represent the mean$\pm$s.e. of 6 independent experiments.

Incubation of CHOhumH$_1$ cells that had been exposed to 100 μM histamine for 24 h, for 10 min with 1 μM PMA resulted in a further attenuation of the 100 μM histamine- and 100 μM ATP-induced Ca^{2+} response (45$\pm$4% and 43$\pm$6% of the response obtained in histamine (24 h)-treated cells, respectively, $n=3$, mean$\pm$s.e.).

Discussion

In the present study we have shown that the human H_1 receptor expressed in CHO cells encodes a classical histamine H_1 receptor with a pharmacology similar to that of the H_1 receptor found in guinea-pig cerebellum (Ter Laak *et al.*, 1993) and the endogenously expressed human H_1 receptor in 1321N1 astrocytoma cells (Nakahata *et al.*, 1985, present study) (Table 1). The pK_i values of different H_1 receptor antagonists, including their respective enantiomers, are in accordance with the reported pK_i values determined in guinea-pig cerebellum and calculated values found in 1321N1 astrocytoma cells (Table 1). Similar results were recently reported for the human H_1 receptors, expressed in COS-7 and CHO cells respectively (De Backer *et al.*, 1993; Moguilevsky *et al.*, 1994). Although a few studies on H_1 receptor signalling of the cloned guinea-pig (Leurs *et al.*, 1994) and bovine (Iredale *et al.*, 1993) H_1 receptor, both expressed in CHO cells, have been reported, our study is the first describing H_1 receptor signalling after stable transfection of the human H_1 receptor gene. In CHO cells stably expressing the human H_1 receptor (CHOhumH$_1$), histamine induced a concentration-dependent mepyramine-sensitive rise in inositol phosphates and a rapid increase of $[Ca^{2+}]_i$, followed by a sustained increase of $[Ca^{2+}]_i$ upon addition of 100 μM histamine. At present we have no explanation for the discrepancy in results with respect to the mepyramine sensitivity of the two H_1 receptor responses. Taken together, this cell line, expressing a high density of H_1 receptors (861$\pm$41 fmol mg^{-1} of protein) allows for the first time investigation of regulatory phenomena such as receptor down-regulation. As such, the CHOhumH$_1$ cell line may be considered a suitable model system to study H_1 receptor regulation and signalling.

The recent cloning of the guinea-pig (Horio *et al.*, 1993; Traiffort *et al.*, 1994), bovine (Yamashita *et al.*, 1991), rat (Fujimoto *et al.*, 1993) and human H_1 receptor (De Backer *et al.*, 1993; Fukui *et al.*, 1994; Moguilevsky *et al.*, 1994) has revealed several potential phosphorylation sites in the amino acid sequence of the H_1 receptor. Three potential PKC phosphorylation sites are found in the amino acid sequence of the H_1 receptor gene, as well as a potential protein kinase A site. In addition, the third intracellular loop contains many threonine and serine residues that can be potentially phosphorylated by specific receptor kinases. As it is well-known for the family of G-protein coupled receptors, that kinases play important roles

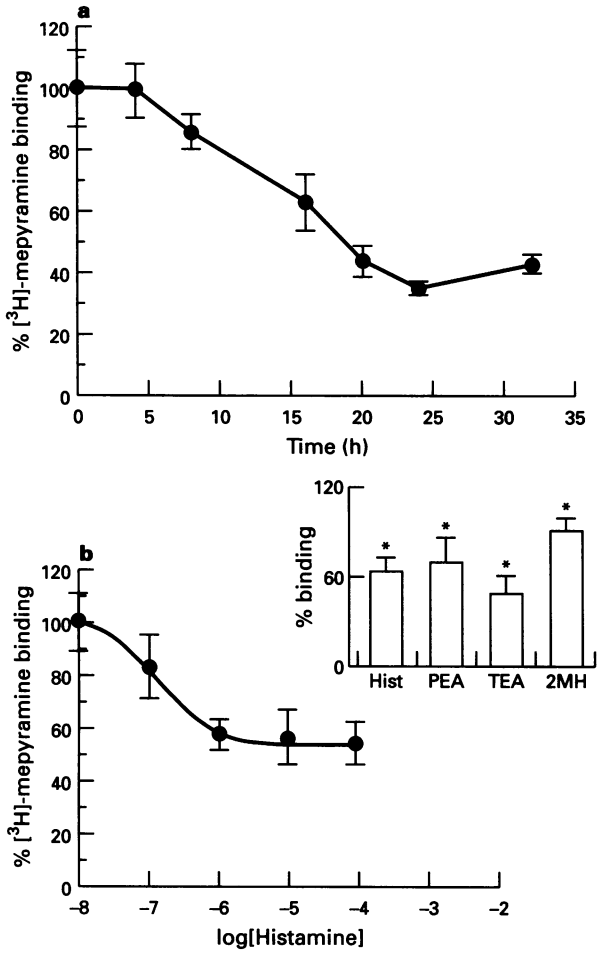

Figure 5 Histamine-induced reduction of [^{3}H]-mepyramine binding in CHOhumH$_1$ cells. (a) CHOhumH$_1$ cells were incubated with 100 μM histamine for the indicated times and [^{3}H]-mepyramine binding in membranes was measured. The [^{3}H]-mepyramine binding is expressed as a percentage of [^{3}H]-mepyramine binding measured in non-treated cells. The data shown represent the mean ± s.e. of 3 independent experiments. (b) Concentration-dependent decrease of [^{3}H]-mepyramine binding induced by histamine. CHOhumH$_1$ cells were exposed to various concentrations of histamine for 24 h. The data represent the mean ± s.e. of 3 independent experiments. (Inset) Effect of pretreatment with H$_1$ agonists on [^{3}H]-mepyramine binding of CHOhumH$_1$ cells. CHOhumH$_1$ cells were incubated for 24 h with histamine (Hist, 100 μM), 2-PEA (PEA, 100 μM), 2-TEA (TEA, 100 μM) and 2-MeHA (2MH, 100 μM). [^{3}H]-mepyramine binding to the membranes was measured as described above. Data were calculated as the mean ± s.e. from at least 3 independent experiments. The asterisks indicate a statistically significant difference from non-treated cells.

in mechanisms such as receptor desensitization and down-regulation (Collins, 1993; Lohse, 1993), all these sites may represent possible targets for phosphorylation.

Previous studies have shown that the histamine H$_1$ receptor is susceptible to short-term receptor desensitization. Depending on the cell type both homologous (Quach *et al.*, 1981; Dillon-Carter & Chuang, 1989; Cowlen *et al.*, 1990; Leurs *et al.*, 1990; Smit *et al.*, 1992; Dickenson & Hill, 1993; McCreath *et al.*, 1994) and heterologous (Brown *et al.*, 1986; McDonough *et al.*, 1988; Bristow & Zamani, 1993; Dickenson & Hill, 1993) H$_1$ receptor desensitization have been observed. In the present study it was shown that short-term exposure of CHOhumH$_1$ cells to histamine resulted in a decrease of subsequent histamine-induced Ca^{2+} responses. The histamine-induced desensitization appeared to be heterologous as the ATP-induced Ca^{2+} response, mediated by interaction with endogenous P_{2U} purinoceptors present on this cell line (Iredale & Hill, 1993; Leurs *et al.*, 1994), was also found to be affected. Moreover, pretreatment of CHOhumH$_1$ cells with ATP also desensitized the histamine-induced Ca^{2+} responses.

The process of heterologous histamine-induced desensitization in CHOhumH$_1$ cells can be ascribed to an alteration at the level of the intracellular Ca^{2+} pool, as reported previously for other model systems (Hishinuma & Uchida, 1988; Leurs *et al.*, 1990; McDonough *et al.*, 1988). The Ca^{2+} response of caffeine, which releases Ca^{2+} from intracellular Ca^{2+} stores (Palade *et al.*, 1989) was attenuated upon short-term histamine exposure. Ineffective Ca^{2+}-pump or Ca^{2+} release mechanisms of the intracellular Ca^{2+} pool might explain the observed findings.

In various cellular systems, PKC has been found to be implicated in negative feedback of H$_1$ receptor signalling (Dillon-Carter & Chuang, 1989; Cowlen *et al.*, 1990; Leurs *et al.*, 1990; Fukui *et al.*, 1991; Smit *et al.*, 1992; Dickenson & Hill, 1993; McCreath *et al.*, 1994). Besides attenuation of H$_1$ receptor signalling, direct activation of PKC was also found to desensitize other phospholipase C-linked receptors present on these cellular systems, implying that PKC induces heterologous desensitization. However, in DDT$_1$MF-2 cells, PKC-independent mechanisms appeared to be involved in the heterologous desensitization of the histamine H$_1$- and ATP-receptor mediated Ca^{2+} responses (Dickenson & Hill, 1993). In CHOhumH$_1$ cells the PKC activator, PMA, was found to inhibit the histamine- as well as the ATP-induced Ca^{2+} response. At a maximally effective concentration of PMA, the inhibition of the response was only partial, and clearly less effective than histamine-pretreatment. In contrast, in for example, cultured astrocytes (Fukui *et al.*, 1991), human umbilical vein endothelial cells (McCreath *et al.*, 1994) and in HeLa cells (Smit *et al.*, 1992) H$_1$-receptor mediated responses have been reported to be highly sensitive to PKC activation. In CHOhumH$_1$ cells pretreatment with the specific PKC inhibitor, Ro-31-8220, did not inhibit the histamine-induced desensitization. Moreover, PKC downregulation induced by long-term exposure to PMA did not affect histamine-induced desensitiza-

Table 2 Effect of long-term PKC and P_{2U}-receptor activation on [^{3}H]-mepyramine binding of CHO humH$_1$ cells

Compounds		*% downregulation*	n
Histamine	100 μM	41 ± 5*	11
PMA	0.1 μM	−13 ± 5	6
PMA	1 μM	−5 ± 5	18
Histamine (100 μM) + PMA (1 μM)		27 ± 3*#	5
ATP	100 μM	15 ± 8	6

CHOhumH$_1$ cells were incubated for 24 h with the indicated compounds and [^{3}H]-mepyramine binding to the membranes was measured. The [^{3}H]-mepyramine binding is expressed as a percentage of downregulation compared to non-treated cells. Data were calculated as the mean ± s.e. from *n* independent experiments. The asterisks and # indicate a significant difference ($P<0.05$) from control, represented by untreated cells, and histamine-treated cells respectively.

tion. Although long-term incubation of CHOhumH_1 cells with 1 μM PMA did not fully downregulate PKC, the remaining PKC-mediated reduction of the histamine-induced Ca^{2+} response cannot explain the observed histamine-induced desensitization. In addition, concurrent incubation of these cells with histamine and PMA resulted in a further attenuation of the histamine-induced Ca^{2+} response. These results indicate that in CHOhumH_1 cells, PKC is probably not involved in the heterologous desensitization.

The discrepancy regarding the role of PKC in the heterologous desensitization between CHOhumH_1 cells and other cellular systems may be explained by differential expression of PKC isoenzymes. PKC is known to consist of a family of different PKC isoenzymes (Nishizuka, 1988). As such, this cell line may be considered as a suitable model system to investigate the role of different PKC isoenzymes in the negative feedback modulation of the human H_1 receptor more closely. Therefore, we stably expressed different PKC isoenzymes cDNAs (α, β_I, β_{II}, δ, ε and γ) (Ono *et al.*, 1987) separately into CHOhumH_1 cells. Although in clonal cell lines expression of 1.4 to 3.4 fold [^{3}H]-PdBu binding was obtained (data not shown), [^{3}H]-mepyramine binding studies revealed disappearance of H_1 receptors on membranes of these clonal CHOhumH_1 cell lines, expressing one of the PKC isoenzymes. Since our co-expression approach was unsuccessful, a detailed knowledge of the distribution of different PKC isoenzymes in the various cellular systems, as well as the future availability of specific PKC isoenzyme inhibitors/activators, could perhaps give more insight in the contribution of each PKC isoenzyme in negative feedback of H_1 receptor signalling.

Thus far, no mechanistic information is available on long-term regulatory mechanisms such as H_1 receptor downregulation. Long-term treatment of CHOhumH_1 cells with histamine or other H_1 agonists resulted in a time- and concentration-dependent decrease in the number of H_1 receptor binding sites. In the neuroblastoma cell line NIE-115 and neuroblastoma/glioma hybrid cell line NG108-15, PKC stimulation with phorbolesters was found to induce downregulation of respectively muscarinic receptors (Liles *et al.*, 1986) and δ-opioid receptors (Gucker & Bidlack, 1992). Yet, neither short-term nor long-term stimulation of the CHOhumH_1 cells with PMA affected H_1 receptor expression. For the δ-opioid receptor, Gucker & Bidlack (1992) showed that simultaneous activation of PKC and the δ-receptor is required to induce receptor downregulation. Yet, no downregulation was observed when cells were treated simultaneously with PMA and histamine for a period of 4 h, indicating that PKC potentiation is apparently not occurring in CHOhumH_1 cells. In addition, concurrent incubation of cells with PMA and histamine for 24 h did not induce a more pronounced H_1 receptor downregulation than with histamine alone but significantly inhibited the histamine-induced H_1 receptor downregulation. Earlier studies in HeLa cells showed an impaired formation of GTPγS-induced inositol phosphate production after PKC activation, indicating effects of PKC on the G-protein (Tilly *et al.*, 1990). As G-protein coupling was found to play an important role in the process of β_2-adrenoceptor downregulation (Campbell *et al.*, 1991) future studies should examine whether G-protein coupling is important for H_1 receptor downregulation.

No significant H_1 receptor downregulation in CHOhumH_1 cells was observed upon long-term ATP exposure, indicating that the H_1 receptor density is not affected upon activation of other receptors coupled to phospholipase C. Thus, stimulation of phospholipase C is not sufficient to induce a reduction of receptor binding sites. These findings are corroborated by the difference in potency for histamine-induced inositol phosphate production and receptor downregulation (EC_{50} 2.23 μM and 0.13 μM, respectively). Interestingly, the EC_{50} for the homologous short-term desensitization of the H_1 receptor in human umbilical vein endothelial cells was also found to be lower than the EC_{50} for inositol phosphate production (McCreath *et al.*, 1994). Taken together, these data imply that H_1 receptor downregulation occurs at the level of the receptor itself, and does not appear to be induced by activation of phospholipase C or PKC.

To study the possible functional consequences of the H_1 receptor downregulation, we also measured the Ca^{2+} responses in histamine-downregulated CHOhumH_1 cells. We observed that long-term histamine exposure of CHOhumH_1 cells also affected receptor-mediated Ca^{2+} signalling. Long-term treatment of CHOhumH_1 cells with 100 μM histamine resulted in attenuation of both the histamine- as well as the ATP-induced Ca^{2+} responses, suggesting that the reduction of the Ca^{2+} responses is not only caused by H_1 receptor downregulation. If PKC stimulation were responsible for the effects of long-term stimulation on the Ca^{2+} responses, one would expect, based on the partial sensitivity of the Ca^{2+} responses towards PMA, that Ca^{2+} responses of histamine-pretreated (24 h) cells would not be further affected by PMA treatment. Nevertheless, PMA still induced a similar reduction of the histamine-induced Ca^{2+} response in CHOhumH_1 cells pretreated for 24 h with histamine, indicating that PKC is not responsible for the observed reduction of the histamine- and ATP-mediated Ca^{2+} responses. Although we have no direct evidence, this reduction may be explained by an alteration at a level distant from the receptor, such as the G-protein or Ca^{2+} pool. Mullaney *et al.* (1993) showed that agonist-induced downregulation of m_1 muscarinic acetylcholine receptors expressed in CHO cells was paralleled by a specific downregulation of cellular levels of the α subunits of G-protein G_q and G_{11} (Mullaney *et al.*, 1993). For other receptor systems, linked to adenylate cyclase, similar observations were made with regard to $G_{\alpha s}$ subunit downregulation (see for references Mullaney *et al.*, 1993). Another possibility is a downregulation of the IP_3 receptor upon long-term receptor activation. In SH-SY5Y human neuroblastoma cells, chronic muscarinic stimulation was found to suppress the Ca^{2+} releasing activity of IP_3, which was paralleled by a reduction in the number of IP_3 binding sites (Wojcikiewicz *et al.*, 1992).

In conclusion, human H_1 receptors expressed into CHO cells are susceptible to receptor regulation. Short-term activation of the human H_1 receptor to histamine leads to a heterologous desensitization of the agonist-induced Ca^{2+} response, which may be explained by alterations at the level of the intracellular Ca^{2+} pool. Long-term exposure of the CHOhumH_1 cells results in a concentration- and time-dependent downregulation of the human H_1 receptor. Both histamine and ATP-induced Ca^{2+} responses are affected upon long-term histamine treatment, indicating alterations at a level distant from the receptor. PKC does not seem to play a role in either the histamine-induced H_1 receptor desensitization or downregulation in CHOhumH_1 cells, which may be explained by differential expression of PKC isoenzymes in this cell line compared to other cellular systems.

Further detailed investigations are required to elucidate the biological mechanism underlying H_1 receptor downregulation. Other phospholipase C-linked receptors, such as the muscarinic m_1 receptor, are also downregulated upon long-term agonist exposure (Shapiro & Nathanson, 1989). Studies have shown that the third intracellular loop is important for the agonist-induced muscarinic m_1 receptor downregulation. The future use of mutant H_1 receptors, should give more insight in the structural requirements of H_1 receptor desensitization and downregulation.

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